RESEARCH HIGHLIGHT

Fanzors: Striking expansion of RNA-guided endonucleases to eukaryotes

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RNA-guided DNA endonucleases are broadly associated with and play critical roles in prokaryotic adaptive immunity-based CRISPR-Cas systems and IS200/605 family transposons. The Feng Zhang lab recently elucidated the phylogeny, enzymatic activity and DNA recognition mechanism of its eukaryotic counterpart Fanzor, while further reprogramming Fanzor for human genome editing applications, thereby highlighting a universal RNA-guided DNA cleavage mechanism spanning from prokaryotes to eukaryotes.

Bacterial and archaeal CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated genes) systems provide adaptive immunity against invading mobile genetic elements by employing diverse CRISPR RNA (crRNA)-guided Cas effectors.[1] DNA-targeting Cas9 and Cas12 systems utilize multi-domain-containing endonucleases to execute RNA-guided site-specific cleavage of dsDNA.[2] Cas12 proteins, that contain a common single RuvC domain, are further subdivided into Cas12a–k subtypes, that share low sequence similarities and exhibit diverse functionalities including crRNA maturation, ribonucleoprotein (RNP) complex composition and target preference[2,3] (Fig. 1a, b). In general, Cas12 forms a RNP complex with either a single crRNA, or with additional *trans*-activating crRNA (tracrRNA), using its single RuvC domain to cleave both strands of target dsDNA in a protospacer adjacent motif (PAM)-dependent manner[2,3] (Fig. 1b–d). Recent studies have uncovered that Cas9 and Cas12 have evolved independently from TnpB-like proteins IscB[4] and TnpB[5] in IS200/IS605 transposons, respectively. A typical IS200/605 transposon consists of subterminal left-end (LE) and right-end (RE) elements and encoded *tnpA* and *tnpB* genes (Fig. 1e). TnpB contains a single RuvC domain and forms a RNP complex with ωRNA (also known as reRNA) transcribed from the transposon RE element[6,7] (Fig. 1f, g). Similar to Cas12, TnpB is guided by ωRNA to bind and cut both strands of target dsDNA containing a target adjacent motif (TAM, analogous to PAM) (Fig. 1h). Owing to its RNA-guided DNA-targeting activity, Cas12 has been widely leveraged for programmable genome editing,[8] while TnpB can also be reprogrammed for genome editing in human cells.[5–7]

Two groups of Fanzors (Fz1 and Fz2) encoded by transposable elements from giant eukaryotic viruses and several eukaryotic genomes, including metazoans, fungi and protists, were reported to be eukaryotic TnpB-like proteins with undefined function.[9] The recent mechanistic studies on TnpB raise the possibility that Fanzors may act as eukaryotic counterparts of RNA-guided endonucleases. Saito et al.[10] performed phylogenomic analysis and discovered Fanzor branches emerging from TnpB in more diverse eukaryotic species. The Fanzor locus contains an encoded *Fz* gene flanked by conserved LE and RE elements (Fig. 1i). Predicted structures indicated that Fanzors contain WED and RuvC domains (Fig. 1j), implying potential RNA-guided DNA endonuclease activity.

Analysis of soil fungus *Spizellomyces punctatus* SpuFz loci, together with binding and small RNA sequencing experiments, revealed an 88–90 nt ωRNA consisting of a 75 nt conserved region transcribed from the right region of the Fanzor locus and a 14–15 nt variable region at the 3′-end, together acting as a ωRNA (Fig. 1i). Additional ωRNAs were identified from other species using the same approach and found to form RNP complexes with Fanzors, as observed in TnpB systems.[5] Subsequent in vitro cleavage assays showed that Fanzors exhibit TAM preference upstream of the target sequence and generate double-strand breaks by cutting at specific sites on the two DNA strands. Unlike TnpB[7] and Cas12,[2] all tested Fanzors did not show collateral non-specific cleavage activity in trans on dsDNA, dsRNA, ssDNA or ssRNA when bound with target dsDNA. Saito et al. also detected DNA insertion and deletion (indel) efficiency of four Fanzors in human cells, three of which exhibited comparable overall editing efficiency to that of AsCas12f1. Further optimization efforts that focused on SpuFz1 by engineering both ωRNA and SpuFz1 protein enabled improvement of the editing efficiency, providing a new strategy for human genome editing.

Next, Saito et al. undertook cryo-EM studies on SpuFz1 in complex with ωRNA and target dsDNA (Fig. 1k). SpuFz1 adopts a bilobed architecture consisting of REC and NUC lobes (Fig. 1j, k), a common feature observed also in Cas12[2,3] (Fig. 1b, c) and TnpB[6,7] (Fig. 1f, g). The REC and WED domains in the REC lobe bury the TAM-containing DNA duplex, while the guide RNA–target DNA heteroduplex is accommodated by the REC and RuvC domains. The stem1 and partial stem2 of the ωRNA scaffold form extensive interactions with the WED, RuvC and NUC domains, with no contact forming between the core-distal region of stem2 and SpuFz1. Based on structural information, a more compact ωRNA with truncated stem2 was designed and tested for human genome editing, with the single RuvC domain responsible for DNA cleavage (Fig. 1l). A putative water molecule was coordinated simultaneously by the sidechain of a key catalytic residue in the active site of the RuvC domain and a phosphate group in stem-loop1 of ωRNA, indicating that ωRNA may stabilize the active site and contribute to DNA cleavage.

Structural comparisons amongst AsCas12a, ISDra2 TnpB and SpuFz1 show distinct features within each family including tertiary structures of RNA scaffolds and domain architecture and composition

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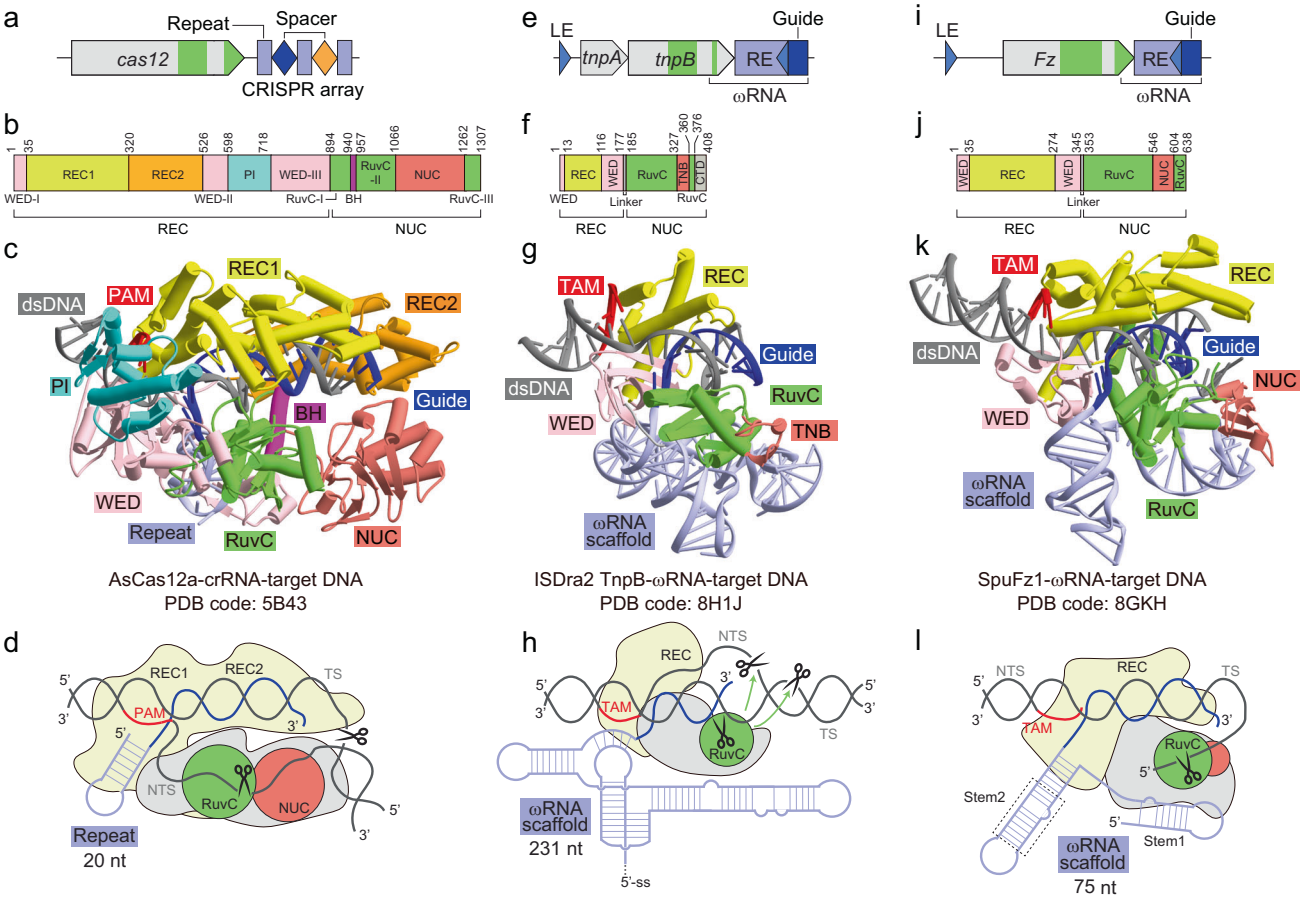

Fig. 1 Structural and mechanistic comparison of Fanzor with Cas12a and TnpB. a, **e**, **i** Schematic diagrams of the CRISPR-Cas12 (**a**), IS200/IS605 transposon (**e**), and Fanzor (**i**) loci. The potential RuvC domains are indicated in green. The CRISPR repeats are represented as light blue rectangles and spacers are represented as differently colored diamonds (blue and orange) (**a**). The LE and RE elements of the transposon, the guide elements and ωRNA are indicated (**e**, **i**). **b**, **f**, **j** Domain organization of *Acidamanococcus sp*. AsCas12a (**b**), *Deinococcus radiodurans* ISDra2 TnpB (**f**), and *Spizellomyces punctatus* SpuFz1 (**j**). **c**, **g**, **k** Structural comparisons among AsCas12a (**c**), ISDra2 TnpB (**g**), and SpuFz1 (**k**) in complex with the corresponding crRNA/ωRNA and target dsDNA. The structures are aligned based on the guide:target heteroduplex. **d**, **h**, **l** Proposed DNA recognition and cleavage mechanisms. The REC and NUC lobes are colored in light yellow and silver, respectively. The active sites of RuvC domains are indicated by black scissors. The region in stem2 of SpuFz1 ωRNA removed for optimization is indicated by a dashed box (**l**). TS, target DNA strand. NTS, non-target DNA strand.

of proteins, while emphasizing a universal RNA-guided DNA targeting mechanism. They employ the REC lobe for PAM/TAM recognition and the NUC lobe for DNA cleavage (Fig. 1d, h, l). Specially, AsCas12a contains a large REC domain consisting of REC1 and REC2 components, as well as an additional PI domain within the WED domain (Fig. 1b, c). ISDra2 TnpB (Fig. 1f, g) and SpuFz1 (Fig. 1j, k) exhibit smaller molecular weights and contain REC and WED domains of reduced size. By contrast, when compared with the crRNA repeat region, ωRNA scaffolds in ISDra2 TnpB and SpuFz1 present large and complex architectures that functionally supplant some domains of Cas12a, suggesting partial replacement by RNA of its protein counterpart during the co-evolution of these two elements.

Collectively, Saito et al. identified and characterized eukaryotic RNA-guided DNA endonuclease Fanzors, thereby demonstrating the universality of the RNA-guided DNA targeting mechanism across all kingdoms of life. Additionally, they established Fanzor-based methods for human genome editing. Two recent preprints also reported on the diversity, biochemical characterization and human genome editing activities of Fanzors.[11,12] Amongst remaining issues to be addressed, the displacement and cleavage mechanism for non-target DNA strand remains unclear, while the biological role of Fanzors in transposition remains mysterious. Additional structural and functional exploration hold future promise for gene editing, as well as other technologies, beyond indel generation.

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ADDITIONAL INFORMATION

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